REMARKS

Claims 1-20, 103, and 114-147 are pending in the present application. Claims 1-20 have been withdrawn by the Examiner as being directed to a non-elected invention. Applicants will cancel these claims upon indication of allowable subject matter.

Applicants note with appreciation the withdrawal of all previous grounds of rejections, and that the Examiner has made the current Office Action **NON-FINAL**.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 103 and 114-147 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement.

Specifically, the Office Action asserts that "...the specification defines 'coiled-coil dimerization domain' as being exemplified by leucine zippers from naturally occurring sources, such as Fos or Jun, or as artificial or synthetic leucine zippers. However, the instant specification does not disclose any other types of 'coiled-coil dimerization domain.' Accordingly, the written description in the instant specification only sets forth leucine zipper dimerization domains as being the only type of 'coiled-coil dimerization domain' that the artisan would be able to recognize as being in Applicant's possession at the time of the invention..."

Applicants respectfully traverse.

First of all, contrary to the Office Action's assertion, the specification itself has described not only the leucine zipper-type coiled-coil dimerization domains, but also the transmembrane regions of HLA-DR α and β chains, which also assemble into coiled-coil structures (see page 33, lines 21-24, citing Cosson and Bonifacino, *Role of Transmembrane Domain Interactions in the Assembly of Class II MHC Molecules, Science* **258(5082)**: 659-62, 1992). These coiled-coil motifs in the MHC Class II proteins are apparently <u>not</u> the short leucine zippers found in transcription factors either. Thus, Applicants have provided adequate written description in the specification for a number of representative species of coiled-coil domains (<u>including</u> and <u>in addition to</u> the leucine zippers).

Secondly, pursuant to MPEP 2163, Section II(A)(2), "[t]he analysis of whether the specification complies with the written description requirement calls for the examiner to compare the scope of the claim with the scope of the description to determine whether applicant has

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demonstrated possession of the claimed invention. <u>Such a review is conducted from the</u> <u>standpoint of one of skill in the art at the time the application was filed</u> (see, *e.g.*, *Wang Labs. v.* *Toshiba Corp.*, 993 F.2d 858, 865, 26 USPQ2d 1767, 1774 (Fed. Cir. 1993)) <u>and should include</u> <u>a determination of the field of the invention and the level of skill and knowledge in the art</u>. Generally, <u>there is an inverse correlation between the level of skill and knowledge in the art and</u> <u>the specificity of disclosure necessary to satisfy the written description requirement. Information</u> <u>which is well known in the art need not be described in detail in the specification</u>. See, *e.g.*, *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379-80, 231 USPQ 81, 90 (Fed. Cir. 1986)." (emphasis added).

Applicants submit that the level of skill and knowledge in the art regarding the coiled-coil domain is very high. Thus, although Applicants have provided adequate written description in the specification for a number of representative species of coiled-coil domains, in view of the level of skill and knowledge in the art, there is really no need to describe the coiled-coil domain in detail in the specification. Thus the written description requirement is met.

As a skilled artisan would appreciate, the characteristic structural feature of the coiled-coil domain is well-known in the art. For example, it is described in detail in one of the widely-used textbook for elementary graduate-level biology course – *Molecular Biology of the Cell*, *Second Edition*, Bruce Alberts *et al.*, Ed., Garland Publishing, Inc., New York, NY (copyright 1989). Copies of certain relevant sections of the textbook are submitted herewith as **Exhibit A**.

On page 618, Figure 11-11 depicts the detailed topology of a coiled-coil, including the alpha-helix structure of an individual coil, the characteristic stripe of hydrophobic ("a" and "d") amino acids and their pattern of winding down around the alpha-helix, and the structure of the coiled-coil itself. Page 617 indicates that "[m]yosin tails, like <u>many</u> cytoskeletal proteins, are long rodlike molecules. The rigid structure of <u>these proteins</u> depends on a <u>common structural</u> <u>motif</u> in which two α helices with a <u>characteristic spacing of hydrophobic residues</u> coil around each other to form a coiled-coil (Figure 11-11)." (emphasis added). Obviously, the coiled-coil domains found in these *cytoskeletal* proteins are not the same as those in leucine zipper, which is commonly found in transcription factors (*e.g.*, *nuclear* proteins).

Also see pages 663-664, "...<u>all</u> cytoplasmic IF proteins are encoded by members of the same multigene family. Their amino acid sequences indicate that <u>each</u> IF polypeptide chain contains a homologous central region of about 310 amino acid residues that forms an extended α

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helix with three short non-α-helical interruptions (Figure 11-74). Moreover, long stretches of this central region have the <u>distinctive sequence motif</u> of a polypeptide that forms a <u>coiled coil</u>." (emphasis added). Such intermediate filament (IF) proteins include at least those large protein families such as: keratins, desmin, vimentin, glial fibrillary acidic protein, neurofilament proteins, and nuclear lamins (see Figure 11-74 on page 663).

Therefore, a large number of <u>known</u> proteins contain the well-known coiled-coil structure.

In addition, the specification cited Oas and Endow (*TIBS* 19: 51-54, 1994), a copy of which is submitted as **Exhibit B**. The first two paragraphs of Oas and Endow indicate that coiled coil domain was first found in the fibrous cytoskeletal proteins such as keratin, tropomyosin, myosin about fifty years ago, in the 1950's. But at around early 1990's (about 5-10 years prior to the filing of the instant application), the "ubiquity" of the coiled-coil domain has emerged in "many diverse proteins." The third paragraph of Oas and Endow states: "...it is <u>well established</u> that static coiled coils serve as dimerization (or multimerization) interfaces in structural proteins" (emphasis added). In fact, the structure of the coiled-coil domain has been so well-characterized by the early 1990's (see the first 12 lines of the second paragraph of Oas and Endow), computer algorithms have been developed to recognize coiled-coil motifs in several proteins that were not previously thought to contain coiled-coil structure. See the first paragraph of Oas and Endow on page 53, citing the COILS program and three references.

All these would not be possible if the structural features common to all coiled-coil domains are unclear to a skilled artisan.

Furthermore, the level of skill and knowledge in the art regarding the coiled-coil domain is so high, that a skilled artisan can not only <u>predict</u> the presence of coiled-coil domains in many proteins, but also <u>actively design</u> artificial coiled-coils that function as dimerization domains. See the bottom of page 33 of the instant specification, citing Pack and Pluckthun (*Miniantibodies: Use of Amphipathic Helices to Produce Functional, Flexibly Linked Dimeric FV Fragments with High Avidity in Escherichia coli. Biochemistry* **31(6)**: 1579-84, 1992).

Therefore, contrary to the Office Action's assertion, Applicants have provided adequate written description for a number of representative coiled-coil domains, despite the very high level of skill and knowledge in the art. The skilled artisan can immediately envision not only the natural or artificial leucine zipper dimerization domains typically found in transcription factors,

but also the other well-known coiled-coil domains found in a great number of cytoskeletal proteins (such as various IF proteins), motor proteins (such as myosins), MHC Class II molecules, viral hemagglutinin (see Oas and Endow), and heat shock proteins (see Oas and Endow), just to name a few. Thus the written description requirement is met in all pending claims. Reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph are respectfully requested.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue.

The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition therefor and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945**, under order No. HUIP-P01-005.

Dated: September 2, 2005   Respectfully submitted,

By _Luka Vauna per_

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EXHIBIT A

|← 37 nm →|

actin monomer
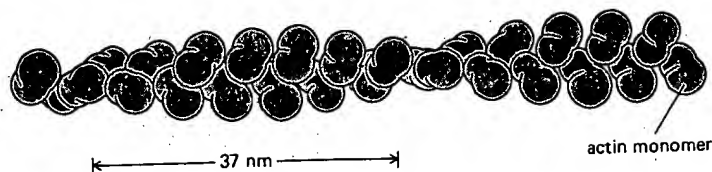
of thin filaments in skeletal muscle by their appearance in the electron microscope, their x-ray diffraction pattern, and their staining with anti-actin antibodies. The thin filaments of muscle are composed of more than just actin, however, as described below (see p. 622).

Actin filaments consist of a tight helix of uniformly oriented actin monomers (Figure 11–7). They are polar structures, with two structurally different ends. The polarity is essential to their function in cell motility and is most readily detectable by the oriented complex that each monomer in the filament forms with myosin. Before discussing this crucial interaction, however, we need to consider some of the properties of myosin molecules.

Thick Filaments Are Composed of Myosin[2]

Myosin is found in almost every cell type in the vertebrate body and is always present where actin filaments form contractile bundles in the cytoplasm. It has been much less highly conserved in evolution than actin and occurs in several forms. The filaments formed when skeletal muscle myosin polymerizes spontaneously *in vitro*, for example, are much larger than the assemblies formed *in vitro* by nonmuscle cell myosins.

Myosin can be specifically extracted from skeletal muscle by treatment with concentrated salt solutions, which causes the thick filaments to depolymerize into their constituent myosin molecules (Figure 11–8). Each molecule consists of six polypeptide chains: two identical *heavy chains* and two pairs of *light chains* (Figure 11–9).

The proteolytic enzyme papain cleaves the myosin molecule into a long α-helical section, called the *myosin rod* (or *myosin tail*), and two separate globular *myosin heads*, also called *subfragment-1*, or *S1 fragments* (Figure 11–10). These two parts of the myosin molecule mediate different functions: the tail is responsible for the spontaneous assembly of myosin molecules into thick filaments, whereas the heads are responsible for moving the molecules against adjacent actin filaments. We shall first discuss the structure and spontaneous assembly of the tails and then consider how the heads generate force.
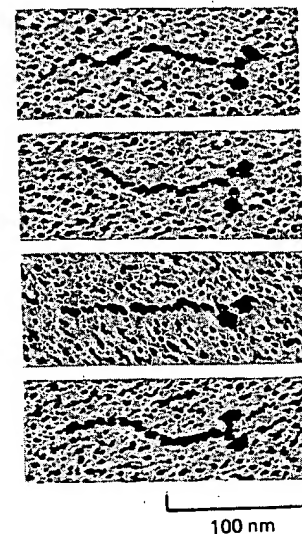

|_____|
100 nm

11-4 ## Myosin Tails Assemble Spontaneously into a Bipolar Thick Filament[3]

Myosin tails, like many cytoskeletal proteins, are long rodlike molecules. The rigid structure of these proteins depends on a common structural motif in which two α helices with a characteristic spacing of hydrophobic residues coil around each other to form a **coiled coil** (Figure 11–11). In myosin, and many other cytoskeletal proteins, the two helices run in parallel (that is, in the same direction from amino to carboxyl terminal) to give rise to a filament that has a diameter of about 2 nm.

While the structure of individual myosin molecules depends on hydrophobic interactions between the two α-helical heavy chains (see Figure 11–11A), the structure of the thick filaments that myosin molecules form in muscle depends on

coiled coil of two α helices

2 nm [

C terminus

light chains

hinge region

N terminus

|← 150 nm →|
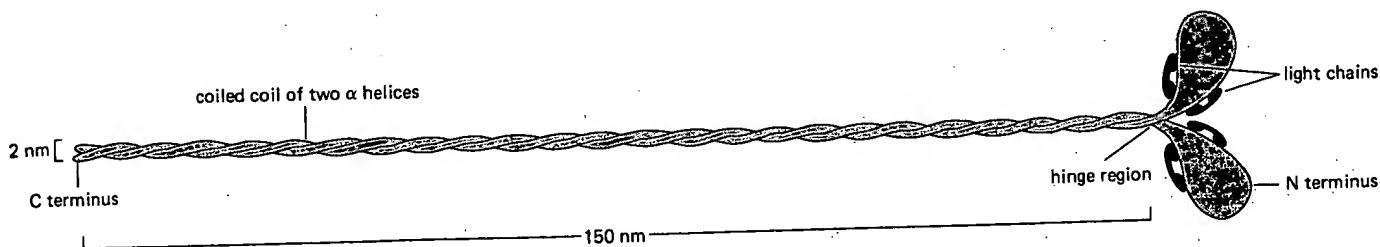
ionic interactions between the tails of the individual molecules. This is why solutions of high salt concentration, which disrupt ionic interactions but do not affect hydrophobic interactions, release individual myosin molecules from muscle. As the salt concentration is reduced to physiological ionic strength, the tails of the myosin molecules associate to form large filaments that may closely resemble muscle thick filaments. In muscle cells these interactions are stabilized by various accessory proteins, and the thick filaments that form are composed of hundreds of myosin tails packed together in a regular staggered array from which the myosin heads project in a repeating pattern (Figure 11–12). The structure is bipolar, with a bare central region where two oppositely oriented sets of myosin tails come together. The globular heads of the myosin molecules interact with actin, forming the cross-bridges between the thick and thin filaments.

Figure 11–10 Limited digestion with the proteolytic enzyme papain cleaves the myosin molecule into a rod and two heads.

11-5 ATP Hydrolysis Drives Muscle Contraction[4]

Skeletal muscle converts chemical energy into mechanical work with very high efficiency—only 30% to 50% of the energy is wasted as heat. (An automobile engine, by contrast, typically wastes 80% to 90% of the energy available from gasoline.)

The energy for muscle contraction comes from ATP hydrolysis. Yet one detects no major difference in ATP levels between a resting muscle and one that is actively contracting, because a muscle cell has a very efficient backup system for regenerating ATP. The enzyme *phosphocreatine kinase* catalyses a reaction between an even more reactive phosphate compound, *phosphocreatine* (Figure 11–13), and ADP to form creatine and ATP. It is the intracellular level of phosphocreatine that drops after a short burst of muscle activity, even though the contractile machinery itself consumes ATP. The pool of phosphocreatine acts like a battery—storing ATP energy and recharging itself from the new ATP that is generated by cellular oxidations when the muscle is resting.
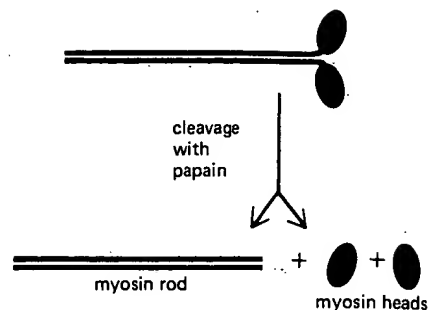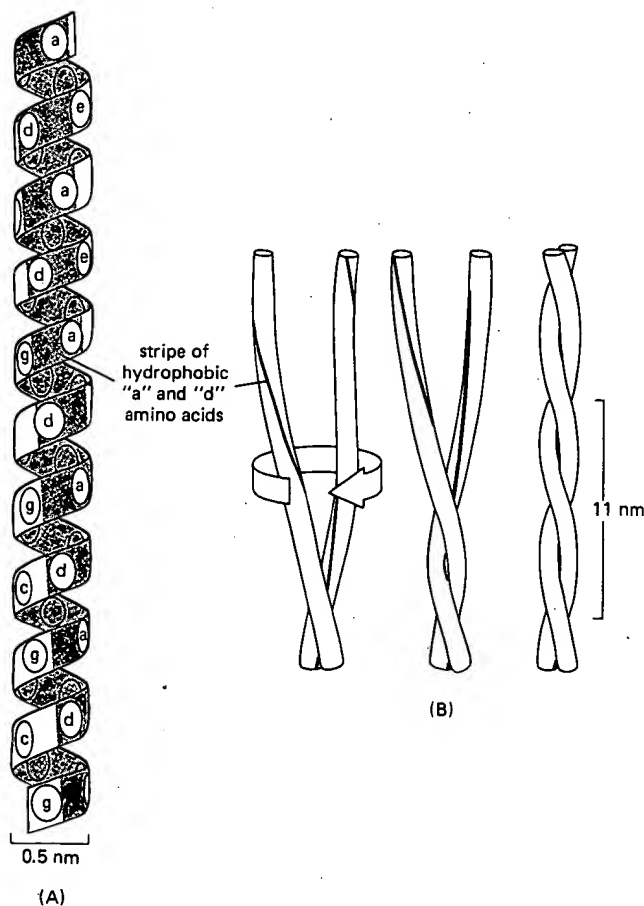

Figure 11–11 Topology of a coiled coil. In (A) a single α helix is represented as a cylinder with successive amino acid side chains labeled in a sevenfold sequence "abcdefg" (from bottom to top). Amino acids "a" and "d" in such a sequence lie close together on the cylinder surface, forming a "stripe" that winds slowly around the α helix (shaded in color). Proteins that form coiled coils typically have hydrophobic amino acids at positions "a" and "d." Consequently, as shown in (B), the two α helices can wrap around each other with the hydrophobic side chains of one α helix intercalated in the spaces between the hydrophobic side chains of the other, while the more hydrophilic amino acid side chains are left exposed to the aqueous environment.

(A)   (B)
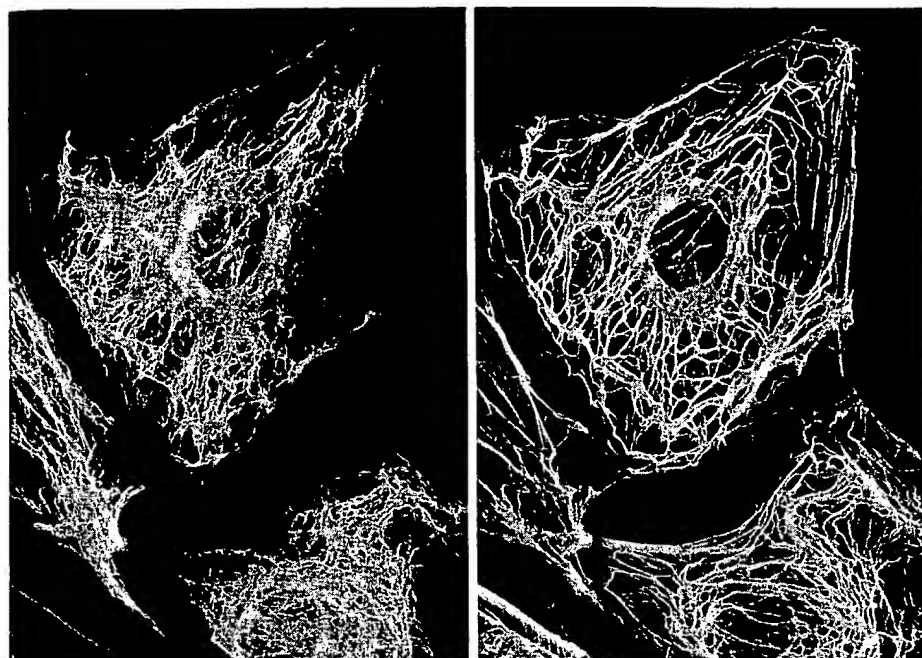
20 μm

Figure 11–73 Immunofluorescence micrographs of rat kangeroo epithelial cells (PtK2 cells) in interphase. The cells have been labeled with antibodies to vimentin (A) as well as with antibodies to keratin (B). Note that the cells contain separate arrays of vimentin filaments and keratin filaments, although the two arrays have a similar distribution. (Courtesy of Mary Osborn.)

to form homopolymers and will also co-assemble with the other Type II IF proteins to form heteropolymers. Indeed, co-polymers of vimentin and desmin, or of vimentin and glial fibrillary acidic protein, are found in some types of cells in tissues.
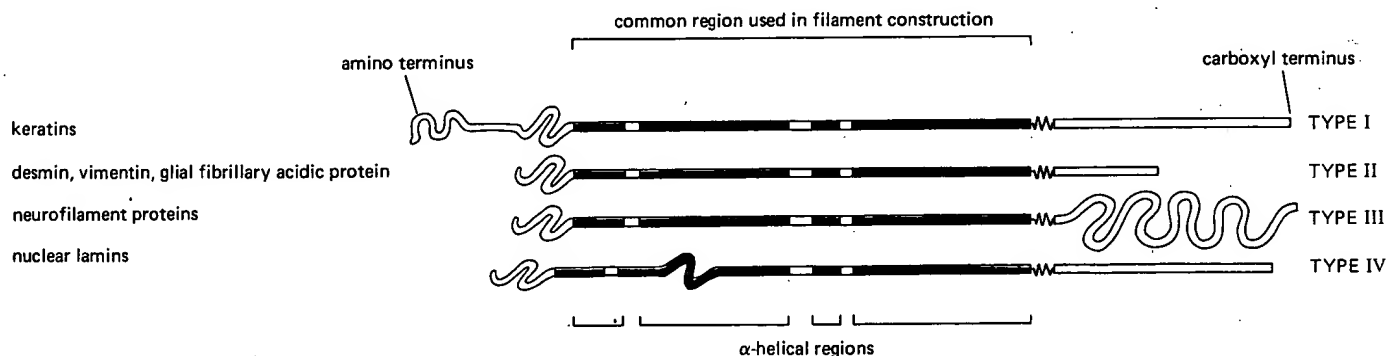
Type III IF proteins assemble into *neurofilaments,* a major cytoskeletal element in nerve axons and dendrites, and consequently are called **neurofilament proteins.** They consist of three distinct polypeptides in vertebrates, the so-called neurofilament triplet. The *type IV* IF proteins are the **nuclear lamins** (see p. 665), which have an amino acid sequence similar to the other IF proteins but differ from them in several ways. Most notably, the lamins form highly organized two-dimensional sheets of filaments, which rapidly disassemble and reassemble at specific stages of mitosis.

All eucaryotic cells make nuclear lamins and usually at least one type of cytoplasmic IF protein. Some cells make two types of cytoplasmic intermediate filaments, which form separate arrays in the cell. Some epithelial cells, for example, have distinct arrays of keratin and vimentin intermediate filaments (Figure 11–73).

11-28 Intermediate Filaments Are Formed from a Dimeric Subunit with a Central Rodlike Domain[53]

Despite the large differences in their size, all cytoplasmic IF proteins are encoded by members of the same multigene family. Their amino acid sequences indicate that each IF polypeptide chain contains a homologous central region of about 310 amino acid residues that forms an extended α helix with three short non-α-helical interruptions (Figure 11–74). Moreover, long stretches of this central region have

Figure 11–74 All IF proteins share a similar central region (about 310 amino acid residues) that forms an extended α helix with three short interruptions. The amino-terminal and carboxyl-terminal domains are non-α-helical and vary greatly in size and sequence in different IF proteins.

common region used in filament construction

amino terminus   carboxyl terminus

keratins   TYPE I

desmin, vimentin, glial fibrillary acidic protein   TYPE II

neurofilament proteins   TYPE III

nuclear lamins   TYPE IV

α-helical regions

the distinctive sequence motif of a polypeptide that forms a coiled coil (see p. 618). As in tropomyosin or the tail of muscle myosin, the coiled coil is double-stranded, with two identical IF polypeptides contributing to form a dimer. The two polypeptide chains in an IF homodimer line up in parallel to form a central rodlike domain with two globular domains at each end. In assembling into an intermediate filament, the rodlike domains interact with one another to form the uniform core of the filament, while the globular domains, which vary in size in different intermediate filament proteins, project from the filament surface. One view of the assembly of the dimeric subunits into intermediate filaments is shown in Figure 11-75.
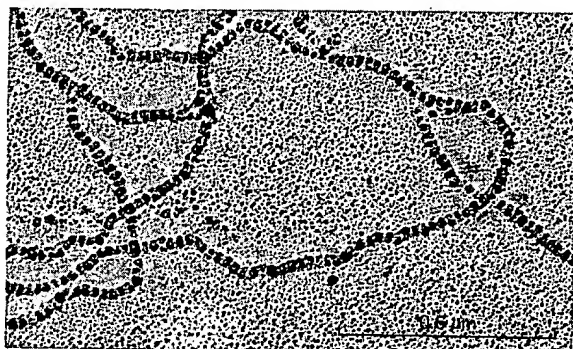

Figure 11-75 A current model of intermediate filament assembly. The monomer shown in (A) pairs with an identical monomer to form a dimer (B) in which the conserved α-helical central regions are aligned in parallel and are wound together into a coiled coil. Two dimers then line up side by side to form a 48-nm by 3-nm *protofilament* containing four polypeptide chains (C). These protofilaments then associate in a staggered manner to form successively larger structures (D and E). The final 10-nm diameter of the intermediate filament is thought to be composed of 8 protofilaments (32 polypeptide chains) joined end on end to neighbors by staggered overlaps to form the long ropelike filament (F). An electron micrograph of the final filament is shown above. It is not known whether intermediate filaments are polar structures (like actin and tubulin) or nonpolar (like the DNA double helix), or whether the two coiled coils in the four-chain protofilament are aligned in a parallel (polar) or an antiparallel (nonpolar) orientation. (Micrograph courtesy of N. Geisler and K. Weber.)
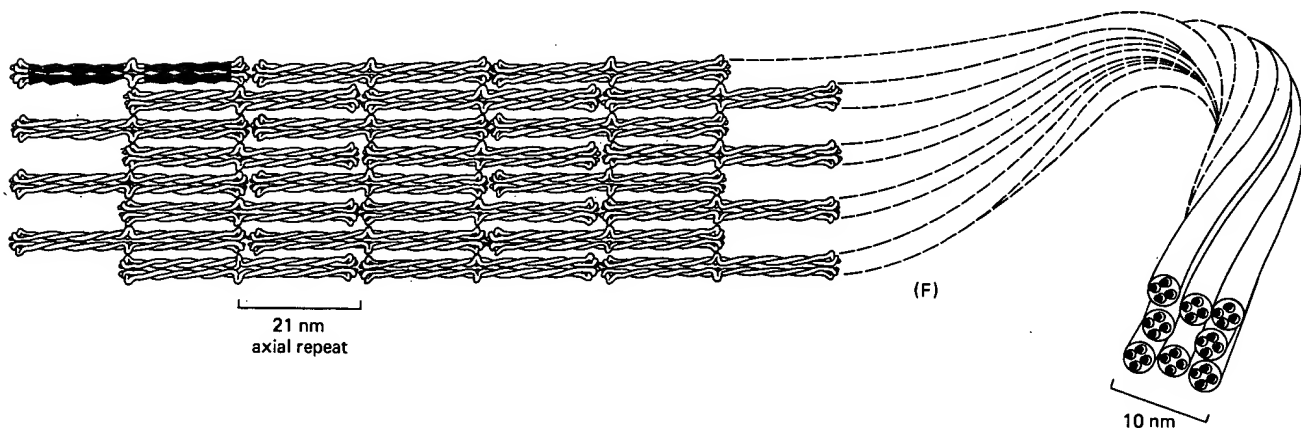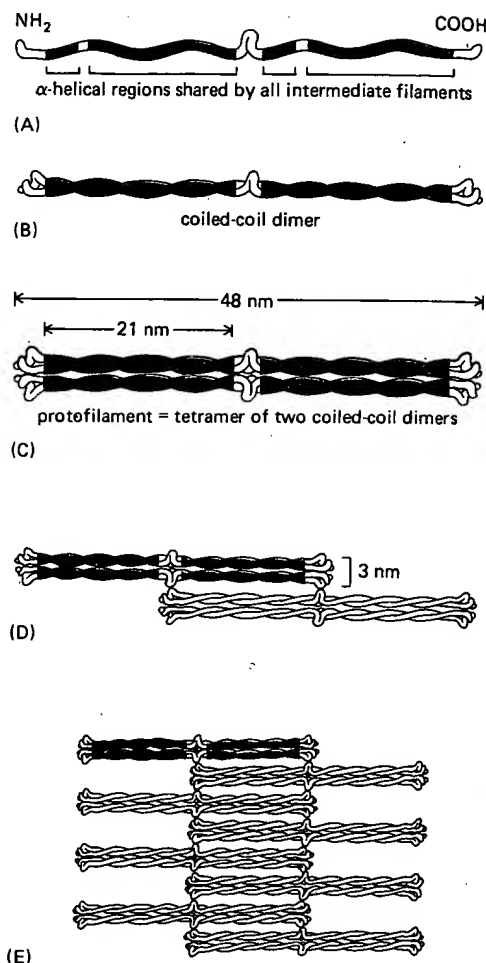
Springs and hinges: dynamic coiled coils and discontinuities

The folding of two or more α-helices into a coiled coil was first postulated for keratin[1,2] and subsequently found in tropomyosin, myosin and other fibrous cytoskeletal proteins. However, the ubiquity of coiled coils in proteins has emerged relatively recently with the isolation and sequence analysis of genes for many diverse proteins, and the development of computer algorithms for recognizing coiled-coil structure from protein sequences.

Nonpolar or hydrophobic amino acids were suggested by Crick[2] to facilitate coiled-coil formation, and he also predicted that nonpolar amino acids would occur, on average, every 3.5 residues in coiled-coil proteins. The characteristic seven-residue repeats, $(a-b-c-d-e-f-g)_n$, with hydrophobic residues in the a- and d-positions, were subsequently recognized as a feature of coiled-coil proteins[3,4]. Leucine is frequently present in the a- and d-positions of heptad repeats from dimeric coiled coils, where it occurs in 27% of all a-positions and 41% of all d-positions analysed[5]. The preference for leucine in the a- and d-positions was first observed[6] for transcription factors such as Fos, Jun and GCN4. Recent studies with GCN4 coiled-coil variants indicate that the packing of α-helices with leucine at the d-position favors dimer formation, while isoleucine at this position favors trimerization[5].

Although it is well established that static coiled coils serve as dimerization (or multimerization) interfaces in structural proteins, it has only recently become apparent that dynamic coiled-coil formation can play a key role in conformational changes, resulting in dramatic movements of one part of a protein relative to another. The kinetics and energetics of dynamic coiled coils are poorly understood. However, this information may emerge from several recently developed systems[5,7–9]. A structural feature that might underlie the ability of coiled coils to facilitate changes in conformation are discontinuities in the heptad repeats of the coiled coil due to deletions, insertions or out-of-frame residues. Here we review

recent reports of coiled-coil transitions in various proteins and propose an approach for testing for such changes in other coiled-coil proteins of interest.

Dynamic coiled coils

Hemagglutinin. A striking example of a coiled-coil transition that is apparently the basis of a dramatic change in protein conformation has emerged from recent work on the influenza virus hemagglutinin protein[8]. Hemagglutinin is a trimeric envelope glycoprotein, formed from three identical polypeptide subunits, that fuses with the host-cell membrane following entry of the virus by endocytosis. Evidence has existed for several years that a large, irreversible conformational change occurs in the hemagglutinin protein upon membrane fusion[10]. The structural basis of the conformational change has been

elusive, despite the availability of the X-ray crystal structure of hemagglutinin at neutral pH[11,12]. Analysis of the protein sequence by Carr and Kim[8] with a computer program that predicts coiled-coil structure[13] resulted in a very high score for a region of the hemagglutinin protein that was a loop, rather than a coiled coil, in the X-ray crystal structure. Carr and Kim synthesized a peptide that included the predicted coiled-coil region together with adjacent residues. Analysis of the peptide by circular dichroism (CD) spectroscopy indicated that it was unfolded at neutral pH, consistent with the loop structure in the X-ray models. At pH 4.8, however, the peptide showed a CD spectrum characteristic of a highly helical conformation. Sedimentation equilibrium studies showed that the peptide was monomeric at neutral pH, but trimeric at acidic pH. These characteristics of the peptide led to the interpretation that the corresponding region of the trimeric hemagglutinin protein is a loop at neutral pH, but an extended trimeric α-helical coiled coil at slightly acidic pH (Fig. 1). The proposed transition from loop to coiled-coil conformation, described as a 'spring-loaded'
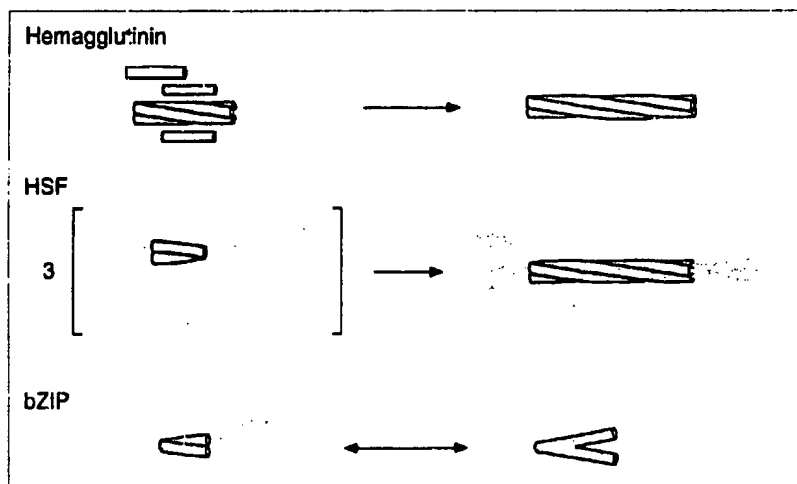

Figure 1

Dynamic coiled coils. Conformational changes into a coiled coil or α-helical structure have been proposed for the following proteins. Top: hemagglutinin; transition of a loop into a coiled coil is induced in the trimeric protein by acidic pH, resulting in an extended coiled coil with the fusion peptide at the tip[8]. Middle: heat shock transcription factor (HSF); a monomer to trimer transition is proposed to be induced by heat shock, resulting in denaturation of an intramolecular coiled coil in the HSF monomer, which then allows formation of a trimeric coiled coil by three HSF monomers[15]. The non-coiled-coil region of the trimer extends to the right, as indicated by the arrows. Bottom: bZIP transcription factor; α-helix formation is induced by binding of the protein to DNA[19]. The region of the protein that undergoes transition into α-helix is shown as a random coil before binding to DNA. The helical fork is the point where the coiled coil separates into two α-helices. Coiled coil is depicted by twisted two- or three-stranded bars, α-helix by cylinders, and non-coiled coil by thick lines. The pitch of the α-helices and the lengths of coiled coils are approximately to scale[26].
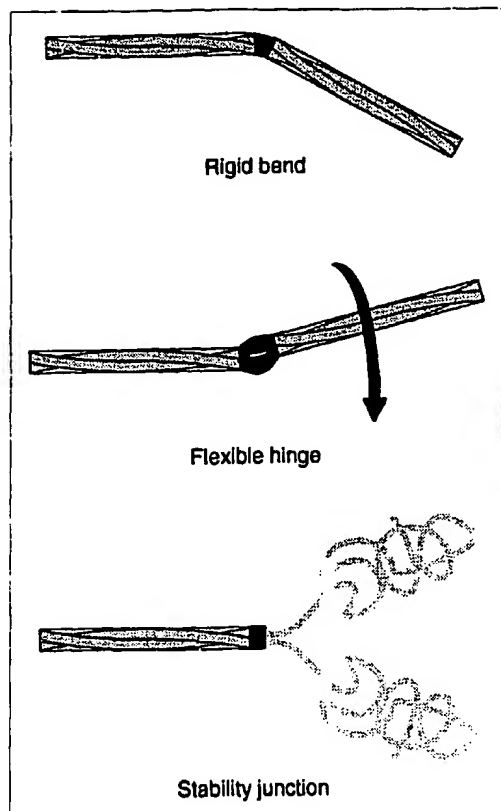
Figure 2

Possible roles of coiled-coil discontinuities in protein function. Discontinuities in a coiled coil may contribute to protein function by top: causing fixed bends, middle: providing flexibility, or bottom: forming boundaries between subdomains of differing stability, or between stable coiled coils and regions of another structure. Coiled coil is depicted by twisted bars and non-coiled coil by thick lines. The pitch of the α-helices is approximately to scale.

mechanism[8], would displace the fusion region of hemagglutinin ~100 Å from a buried position in the protein to an exposed, fusion-competent position.

Comparison of the hemagglutinin sequence with those of other viral fusion proteins indicates that aspects of the conformational change propose] for hemagglutinin might be common to many viral envelope proteins, including that of the type 1 human immunodeficiency virus (HIV-1). Recent experiments with a 38-residue peptide corresponding to the envelope protein gp41, indicate that it forms a coiled coil and, strikingly, the peptide potently inhibits gp41-mediated virus–cell and cell–cell fusion *in vitro*[14]. These results suggest that the peptide competes with intact gp41 for coiled-coil formation with cell-surface proteins.

Heat shock factor. A recent model for the regulation of the human and *Drosophila* heat shock transcription factors (HSF) invokes a dynamic monomer-to-trimer coiled-coil transition[15]. HSF, present in unshocked cells as a monomer, is found after heat shock in an active trimeric form in which it can bind to upstream response elements and activate transcription of heat shock genes. Based on evidence from site-specific mutation and deletion experiments, Rabindran *et al.* propose that trimerization occurs following denaturation, triggered by heat shock, of a coiled coil in the HSF monomer[15] (Fig. 1). Denaturation of the coiled coil of the monomer would allow the formation of a trimeric coiled-coil protein from three HSF monomers. If this model is supported by further experiments, regulation of protein activity can be added to the list of biological functions that can be mediated by coiled-coil transitions.

The bZIP transcription factors. Many dimeric eukaryotic transcription factors contain the 'bZIP' motif, consisting of a small, basic DNA-binding region immediately adjacent to a coiled coil (leucine zipper) which functions as the dimerization interface. Recently, a model has been proposed to explain the mechanism by which bZIP proteins bind to recognition sites in DNA. According to this model, referred to as the 'induced helical fork' model, unbound protein contains an α-helical coiled coil that does not extend into the DNA-binding region of the protein. However, CD studies[16–18] detect an increase in helicity in the presence of DNA, suggesting that coiled-coil helices are extended upon binding (Fig. 1). The forklike point of divergence between the coiled coil and the α-helices allows the α-helices to make specific contacts with bases and phosphates in symmetrical positions of the DNA recognition sequence. The recent crystal structure of the yeast transcription factor GCN4, bound to a recognition sequence, fully supports this model[19]. The structural transition that accompanies binding is the formation of new α-helix, rather than a coiled coil, but it is clear that the coiled-coil region of the bZIP proteins plays a critical role in nucleating and stabilizing the α-helices. In heterodimeric bZIP transcription factors such as Fos–Jun, the coiled-coil transition may be controlled by electrostatic interactions[9,20].

In the examples discussed above, dynamic coiled coils have been postulated to play a role in interaction specificity. A critical component of all of the models described above is the boundary between stable coiled coils and the metastable, dynamic regions adjacent to them. In some cases, these boundaries can be recognized by sequence analysis as discontinuities in the heptad repeats of the coiled coil. Discontinuities are also found in stable coiled coils where they probably perform other functions.

Coiled-coil discontinuities

Many proteins with coiled coils contain one or more discontinuities or interruptions in the heptad repeats that comprise the coiled coil. The inserted, deleted, or out-of-frame residues are sometimes referred to as 'skip' or 'stutter' residues, and are often associated with a phase change in the heptad repeats that follow them. Discontinuities in the myosin-heavy-chain coiled coil have been termed 'hinges'[21]. Recent experiments suggest that coiled-coil discontinuities are needed for normal *in vivo* function of two molecular motor proteins: deletion of regions that include a cytoplasmic myosin hinge or skip residues in a kinesin-related protein cause partial loss-of-function mutant phenotypes[22,23]. Coiled-coil discontinuities could contribute to protein function in different ways – by creating fixed bends in the coiled coil, or by providing flexibility to the molecule (Fig. 2). Alternatively, discontinuities within a coiled coil might be boundaries between subdomains of differing stability, or between coiled-coil and other structure (Fig. 2). The loop of hemagglutinin is an example of a coiled coil whose stability differs across a distinct boundary, while the helical fork in GCN4 and other bZIP transcription factors represents a boundary between coiled-coil and α-helical structure. The definition and classification of coiled-coil discontinuities is useful because they are often easy to detect by sequence analysis.

Identification and functional testing of coiled-coil discontinuities

Discontinuities in a coiled coil can be identified experimentally by local sensitivity to proteases or the presence of a bend in electron micrographs. They can also be identified by the use of computer algorithms that predict coiled coils. The program COILS[13] uses a Parry table (a matrix, based on known coiled-coil proteins, of statistical preferences for amino acids in each position of the heptad repeat) to calculate the probability that a given sequence is a coiled coil and to predict the register, or phase, of the coiled-coil heptad repeat in the sequence. The COILS program has been used to identify coiled coils in several proteins that were not previously thought to contain them[13,24,25]. A further use of this program is to identify discontinuities in coiled-coil sequences. An example of this application is shown using *Dictyostelium* cytoplasmic myosin heavy chain (Fig. 3). The coiled-coil region predicted by COILS is in good agreement with that identified by electron microscopy[22]. Similar predicted discontinuities have previously been detected in other myosin heavy chains with the use of COILS[13].

Careful analysis of the heptad repeat register predicted by COILS for two discontinuities in the coiled coil of cytoplasmic myosin heavy chain, CCD1 and CCD2, immediately suggests mutations that might remove the discontinuities (see Box 1).

The probability of coiled-coil formation for the 'mutated' CCD2 shown in Box 1 is 1.0 (Fig. 3). CCD1 and CCD2 represent deletion and insertion discontinuities. We have also observed several coiled-coil sequences that have replacement discontinuities, which can be removed by replacing residues that are in heptad positions where they are rarely found, with residues that are preferred. For example, Ile, a residue rarely found in heptad position b, could be replaced with the preferred Glu to remove a predicted discontinuity in a coiled coil under study.

Using this approach, coiled-coil discontinuities can be removed conceptually using specific, highly localized mutations. Directed mutagenesis combined with mutant analysis to define phenotypes of the mutated proteins will help to establish the biological role of coiled-coil discontinuities in proteins of interest. Discontinuities can be studied *in vitro* using CD spectroscopy, sedimentation equilibrium centrifugation,
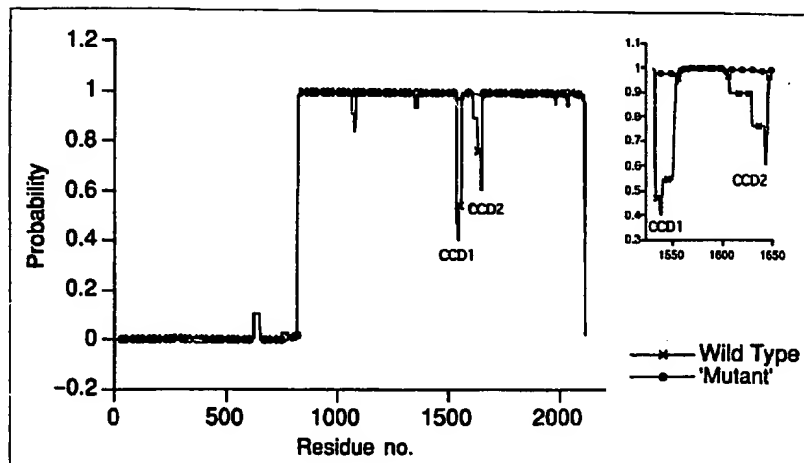

Figure 3

Graph of coiled-coil probabilities versus residue number for *Dictyostelium* cytoplasmic myosin heavy chain, as determined by the program COILS[13]. The probability that residues 820–2110 form a coiled coil is close to one, except for residues 1534–1552 (CCD1) and 1607–1644 (CCD2), where the probability of coiled-coil formation is low. These regions represent discontinuities that might have functional importance (see text for details). CCD1 and CCD2 are shown in more detail in the expanded plot on the right. The effects of the insertion L540E541 on CCD1 and the deletions ΔR629R630A631 and ΔE633I634 on CCD2 are shown as the 'mutant' probability curve.

and other analytical biophysical methods to analyse synthetic peptides or expressed proteins. Synthetic peptides[14], as well as proteins, can also be tested for effects on function *in vivo*. The development of new computer algorithms could eventually allow the prediction of dynamic coiled coils, as well as other classes of coiled-coil discontinuities. Physical studies on model systems may provide important quantitative information for this purpose[5,7].

The combination of the powerful tools of computational and experimental molecular biology to study dynamic coiled coils should give a better understanding of their importance in biological systems.

Box 1. Mutations that remove discontinuities from CCD1 and CCD2

CCD1 has the following sequence and predicted coiled-coil register:

```
efg abcfg abcdefg abcde
FIR KKDAE IDDLRAR LDRET
```

It is apparent from this prediction that one of the heptads is missing two residues at positions d and e. Insertion of Leu (L) and Glu (E), residues frequently found at these heptad positions[4,13], repairs CCD1 to form a continuous heptad repeat:

```
efg abcdefg abcdefg abcde
FIR KKDLEAE IDDLRAR LDRET
```

When this 'mutated' CCD1 sequence is analysed using COILS, the probability of coiled-coil formation becomes 0.97 (see insert in Fig. 3). Similarly, the sequence and register for CCD2 are:

```
cdefg abcdefg abcdefg abcabcdefg abcdefgfg a
TETKS RIKIEKS KKKLEQT LAERRAAEEG SSKAADEEI R
```

The underlined residues can immediately be identified as residues that can be deleted to restore the heptad repeat register for CCD2.

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PH domains and phospholipases – a meaningful relationship?

There have been a number of recent reports concerning a novel domain, the archetype of which is found in pleckstrin, hence the abbreviation PH (pleckstrin homology) domain[1-4]. Among those proteins identified as possessing a PH domain is the β-adrenergic receptor kinase (βARK) and, consistent with the carboxy-terminal location of this domain, is the suggestion that this might confer on βARK its known ability to interact with G protein βγ-complexes[1,3]. This hypothesis will, no doubt, be tested empirically. Nevertheless, it is of interest to consider an effector system known to be responsive to βγ-complexes. The inositol-lipid-specific phospholipase C (PLC) isoforms of the β-subclass are activated by direct interaction with βγ-complexes, as has been established by the reconstitution *in vivo* of purified components (see, for example, Ref. 5). However, PLCβ has not been identified in previous searches for PH-domain-containing proteins. By contrast, PLCγ and PLCδ₂ have been aligned to reveal split (γ) and intact (δ₂) PH domains.

A reappraisal of these sequences, including those of the PLCβ subclass, indicates that, in fact, all retain an intact PH domain at their amino termini (Fig. 1). In particular, this includes the tryptophan residue which is the only residue conserved in all PH domains identified to date. The presence of a discrete domain at the amino terminus of PLC is consistent with proteolytic and mutation studies on PLCδ, which indicate that the first 100 or so amino acids are dispensable for activity and can be cleaved from the catalytic core[6,7]. It should be noted, however, that part of the PLCδ amino terminus might play a role in dimerization[6].
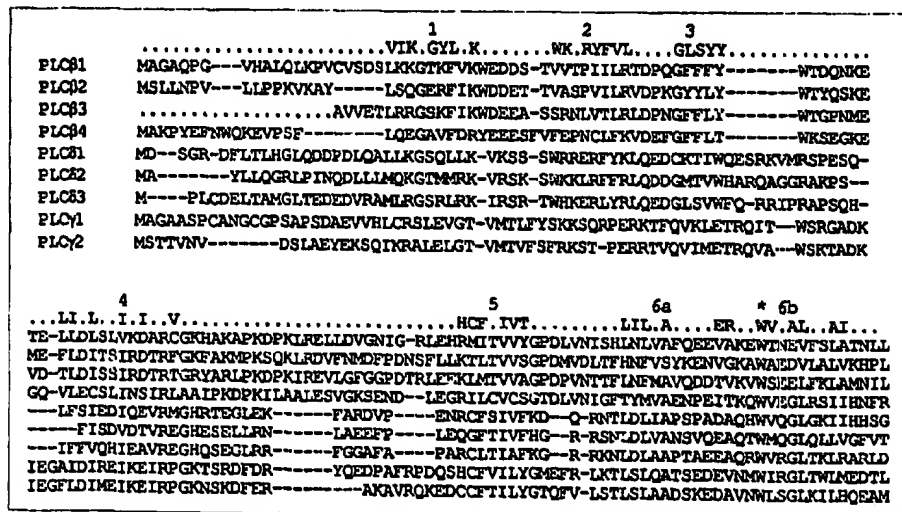

Figure 1
Alignment of the amino-terminal sequences of all the known mammalian PLCs (the full sequence of PLCβ₃ is not yet available). Above the alignment is the PH domain consensus sequence defined by Shaw[3], with subdomains 1-6 indicated. It should be noted that the individual PH-domain sequences do not conform closely to this consensus sequence; the only wholly conserved residue is the tryptophan highlighted with an asterisk.